ORIGINAL ARTICLE 10.1111/j.1469-0691.2006.01515.x

Visual detection of IS*6110* of *Mycobacterium tuberculosis* in sputum samples using a test based on colloidal gold and latex beads

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ABSTRACT

The IS*6110* sequence was detected visually in sputum samples of tuberculosis patients using a bi-probe system. One of the probes was an oligonucleotide conjugated to colloidal gold particles, complementary to one end of the target strand. The other probe was an oligonucleotide conjugated to latex beads complementary to the other end of the target strand. In a reaction mix, these two probes bind to the target strand, and the latex beads are then separated by filtration. Bound latex beads have gold colloid particles at the other end of the target strand. These gold colloid particles were made visible to the naked eye by silver autometallography on the 'invisible' colloidal gold particles. The lower detection limit was 50 ng of genomic DNA of *Mycobacterium tuberculosis*. This new test, together with conventional PCR, was performed on DNA extracted from sputum samples of suspected tuberculosis patients. The new test was simple to perform, the results were visible to the naked eye, and the test was highly specific, as even single point mutations in the target strand sequence could be differentiated. The test could be useful in field-level laboratories because it requires no sophisticated equipment.

Keywords Colloidal beads, diagnosis, IS*6110*, *Mycobacterium tuberculosis*, oligonucleotide probes, visual detection

Original Submission: 17 October 2005; **Revised Submission:** 17 February 2006; **Accepted:** 3 April 2006

Clin Microbiol Infect 2006; 12: 1118–1122

INTRODUCTION

Sequence-specific DNA detection has become increasingly important in the genetic-based diagnosis of disease. Strategies for DNA detection based on functionalised colloidal gold have been described in recent years. Nanoparticle-based detection schemes using two gold particle probes with covalently bound oligonucleotides complementary to a target of interest have also been reported [1,2]. When encountering target strands, these particle probes are polymerised and form network structures, accompanied by a red-to-blue colour change. By incorporating the sophistication of chip technology, improved detection limits and convenience have been achieved. Examples include array-based electrical detection of DNA [3], Raman spectroscopy-based detection [4], magnetic separation followed by chip-based detection [5], and a novel optical detection system [6]. A gold colloid-labelled probe has also been used to detect single point mutations by quenching fluorescence [7]. Zeptomole quantities of nucleic acid have been detected by a colourimetric scatter-based method [8]. Most recently, a detection scheme using non-conjugated gold colloid for DNA has been reported [9]. This technique is based on electrostatic interactions, and detection is made possible by controlling the salt concentration.

The above methods have varying levels of sophistication and, in most reports, only the proof of principle has been demonstrated. Methods in which the final results are read by spectroscopy or chip-based techniques may not be suitable for field-level laboratories. Methods with ultra-low detection limits [8] require stringent precautions to avoid cross-contamination. Similarly, the otherwise simple-to-perform, non-conjugated gold colloid method [9] requires stringent control of the ionic strength of test samples, which may not be easy to achieve when DNA is extracted from clinical samples.

The present study reports a test for detection of specific DNA that is simple to perform, does not

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require any instrumentation to view the final results, and which has been evaluated with relevant clinical samples. The test was developed to detect the IS*6110* sequence of *Mycobacterium tuberculosis*. The potential of this sequence for the diagnosis of tuberculosis (TB) has been demonstrated previously [10–14]. The study first demonstrated the proof of principle by using a synthetic target strand. The sensitivity of the test was then estimated for genomic DNA extracted from *M. tuberculosis*. Finally, the test was evaluated with DNA extracted from sputum samples.

MATERIALS AND METHODS

Two nucleotide probes corresponding to each end of the target strand were synthesised. The exact sequences of these nucleotide probes are shown in Fig. 1. In contrast with PCR, it is not necessary for the two target sequences to be separated by a few bases. It is possible to design appropriate probes to target any unique sequence of ≥ 40 bases; hence, highly specific probes can be made with relative ease. One of the nucleotide sequences was bound covalently to latex beads, while the other nucleotide was linked to colloidal gold particles. Both probes bind to their respective ends of the target strand in a reaction mix. When filtered, the bound and unbound latex beads are retained on the filter. Bound latex beads have gold colloid particles attached at the other end of the target strand, but are still almost invisible on the filter. Direct visual detection of target DNA is made possible by autometallography or silver enhancement [15]. In this process, the DNA captured by functionalised colloidal gold and latex beads is exposed to silver ions containing a reducing agent such as hydroquinone. Gold colloid particles provide nucleation for the deposition of reduced silver from silver ions. During this process the beads become enlarged to 30–100 nm in diameter, and become black in colour.

Preparation and labelling of probes

Gold chloride, $HAuCl_4$ (Sigma, Bangalore, India) was used. Colloidal gold particles with an average diameter of 25–30 nm were prepared by controlled reduction of a 0.02% w/v boiling solution of gold chloride with sodium citrate 1% w/v [16].

The target sequence comprised nucleotides 777–972 of the IS*6110* sequence [17] (GenBank accession no. M29899). To evaluate the assay, the nucleotide sequence shown in Fig. 1, in which nucleotides 807–945 were removed, was synthesised. The target strand was shortened to make its synthesis less expensive.

A-3′-hexylthiol-modified sequence was synthesised based on nucleotides 777–798 of the target sequence; similarly a 5′-hexylamine-modified sequence was synthesised based on nucleotides 952–972 (Fig. 1). In order to evaluate the ability of the test to differentiate single point mutations, a target sequence was synthesised (Fig. 1B) in which the cytosine at position 965 was replaced with thymine. In order to examine the specificity of the test, detection of the target strand was attempted in the presence of a large excess of this 'mutated' strand.

The same nucleotide targets were used to make PCR primers. One primer was similar to the hexylamine-modified nucleotide, while the other was complementary to the hexylthiol-modified nucleotide, but without the hexylamine or hexylthiol modifications.

Conjugation of oligonucleotides to colloidal gold and latex beads. A 200-μL aliquot of colloidal gold solution (see above) was mixed with 10 μL (*c.* 25 ng) hexylthiol-modified nucleotide and incubated overnight at 25°C. Gold-bound nucleotide was washed twice with 1 mL milliQ water and dispensed finally in 200 μL milliQ water.

Carboxylate-modified 0.45-μm latex beads (Sigma) were used for conjugation to the hexylamine-modified nucleotide. A 100-μL aliquot of beads was washed twice in milliQ water. The washed beads were dispersed in 1 mL 10 mM phosphate-buffered saline (PBS) containing 0.1 mg 1-cyclohexyl-3-(2-morphoilino-ethyl)carbodimide metho-p-toluenesulphonate (Sigma), mixed with 50 μL (*c.* 50 ng) hexylamine-modified nucleotide, and incubated overnight at 25°C. Latex beads with bound nucleotide were washed in milliQ water and finally dispersed in 1 mL milliQ water.

For silver enhancement of almost invisible gold nanoparticles, equal volumes of hydroquinone 0.5% w/v and silver acetate 0.2% w/v in 0.5 M citrate buffer (pH 3.8) were used.

Preparation of genomic DNA

Genomic DNA from *M. tuberculosis* (strain H37Rv) was extracted by ethanol precipitation. In brief, glycine 1% w/v was added to the culture the day before harvesting. The cells were centrifuged and resuspended in a lysis solution containing sucrose 10% w/v and lysozyme 2 mg/mL in 50 mM Tris

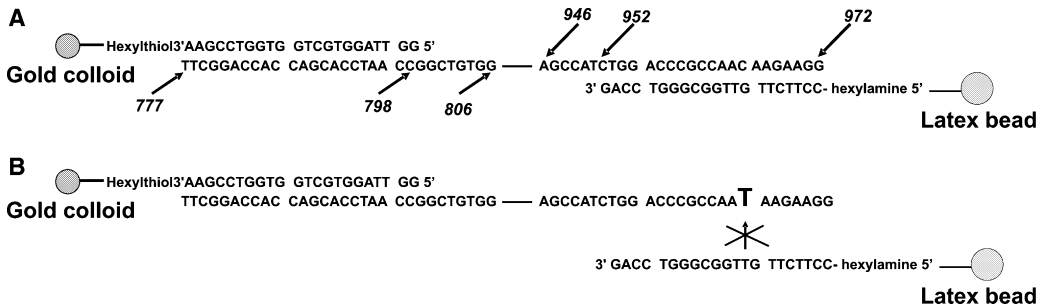

Fig. 1. A, the sequences of the target strand, the nucleotide probe labelled with latex beads, and the nucleotide probe labelled with gold colloid. **B**, after a single mutation in the target strand, the nucleotide labelled with latex beads does not bind. Numbers indicate the position of the sequences in the IS*6110* target sequence.

buffer. Proteinase K and SDS were then added and the mixture was incubated at 55°C. The lysate was extracted with phenol saturated with Tris-EDTA (TE) buffer, the nucleic acid was precipitated with ethanol, dissolved in TE (10 mM Tris, 1 mM EDTA, pH 8) buffer, and the yield was estimated by measuring the absorbance at 260 nm and 280 nm.

Sputum samples

Sputum from suspected TB patients was collected and processed at the New Delhi Tuberculosis Centre, New Delhi, India. The sputum samples were examined initially by microscopy and cultured by Petroff's method.

DNA was extracted from sputum samples by the NALC (N-acetyl L-cysteine) and GITC (guanidium isothiocyanate) method (method A) [18]. Sputum samples were mixed with 0.2 vol. NALC 2.5% w/v in 68 mM phosphate buffer (pH 6.8), allowed to stand for 10 min, and then centrifuged at 10 000 *g*. The pellet was resuspended in inhibitor removal solution containing 5 M GITC, 25 mM EDTA, sarcosyl 0.5% w/v, 0.2 M β-mercaptoethanol in 50 mM Tris-chloride, pH 7.5. After 30 min, the mixture was centrifuged at high speed and the pellet was washed and dried. DNA was extracted from the dried pellet by adding 5 vol. Chelex 100 (Bio-Rad, Bangalore, India) 10% w/v suspension containing Tween 20 0.3% v/v and Triton X-100 0.03% v/v. The tubes were heated at 90°C for 30 min and the supernatant was then used for the PCR and gold colloid–latex bead tests. For gold colloid–latex bead tests, DNA was also extracted (method B) by mixing equal volumes of sputum and NALC 2.5% w/v in 68 mM phosphate buffer for 15 min, centrifuging at high speed, washing the pellet with 10 mM TE buffer, resuspending in 1 mL TE buffer, and then placing the tube over boiling water for 30 min. The supernatant was used for the gold colloid–latex bead test.

PCR assay

Taq PCR core kit (Qiagen, Hilden, Germany) was used to perform the PCR assay, with a hot start for 4 min, followed by 30 cycles of 94°C for 30 s, 62°C for 30 s and 72°C for 30 s. The amplification products were checked on an ethidium bromide-stained agarose gel.

Gold colloid–latex bead test procedure

Single-stranded DNA was prepared by taking 10 μL of DNA sample extracted by any of the above methods, keeping the tube in boiling water for 20 s to denature the DNA, and then placing the tube in a freezing mixture consisting of NaCl and ice for 20 s to achieve rapid cooling and prevent re-annealing. This heating/cooling cycle was repeated three times. Twenty μL of latex beads probe, 10 μL of gold probe and 5 μL of 100 mM PBS were then added to the denatured DNA sample. After 5 min, the reaction mix was filtered through a 0.22-μm polyvinylidene fluoride (PVDF) hydrophilic filter (Millipore, Bangalore, India). Latex beads retained on the filter were washed with 10 mL milliQ water to remove unbound gold probe and chloride ions, after which 50 μL of silver enhancement solution was added. The filter was examined visually after 30 min for black spots. The image intensity was estimated by manually selecting the image area corresponding to each spot and converting it to a grey scale. The colour intensity was then measured with ImageJ software (http://rsb.info.nih.gov/ij/index.html).

RESULTS AND DISCUSSION

The principle of the gold colloid-latex bead test was demonstrated initially with a synthetic target DNA strand. A series of dilutions containing 0–2.5 ng of target was made. These dilutions were mixed with both probes as described above. Fig. 2A shows examples of the filters before and after silver enhancement. Following enhancement, it was possible to clearly distinguish the black spot corresponding to 25 pg DNA from the blank.

Fig. 2B shows a comparison of the results obtained by PCR and the gold colloid–latex bead test. A series of dilutions containing 0–50 μg of genomic DNA extracted from strain H37Rv was made and both tests were performed. Lanes 2–5 and 7 show the 200-bp amplification product from 5 ng, 50 ng, 0.50 μg, 5 μg and 50 μg of target DNA, respectively. The gold colloid–latex bead test was performed on solutions containing the same amounts of DNA (the filters are shown below the respective lanes in Fig. 2B). The spot corresponding to 5 ng DNA is not visible, the 50 ng spot is only just visible, and all other spots are clearly visible. Therefore, the lower detection limit of the gold colloid–latex bead test is 50 ng of H37Rv genomic DNA. This detection limit corresponds to 10^8 cells as there are 16 copies of IS*6110* present in strain H37Rv [19], and a single genome contains 5 fg of DNA.

Fig. 2C shows the average image intensity of five filters, together with the standard deviation. The intensity increased with the amount of target DNA, albeit with a large standard deviation caused by the limitations of silver autometallography. For this reason, quantitative estimation of DNA by the gold colloid–latex bead test may not be possible, but the test could be used to define positive or negative clinical samples.

Applicability of the test for DNA mixtures

To verify the applicability of this test for the detection of the target sequence in a mixture of similar or excess random DNA, the intensity obtained with several types of mixtures was investigated. Fig. 2D shows that 50 pg of target was detected, even in the presence of 0.5 μg genomic DNA from *Escherichia coli* or 50 ng of mutated sequence. These results show that it is possible to adapt this test to detect single point mutations, and that it remains sufficiently sensi-

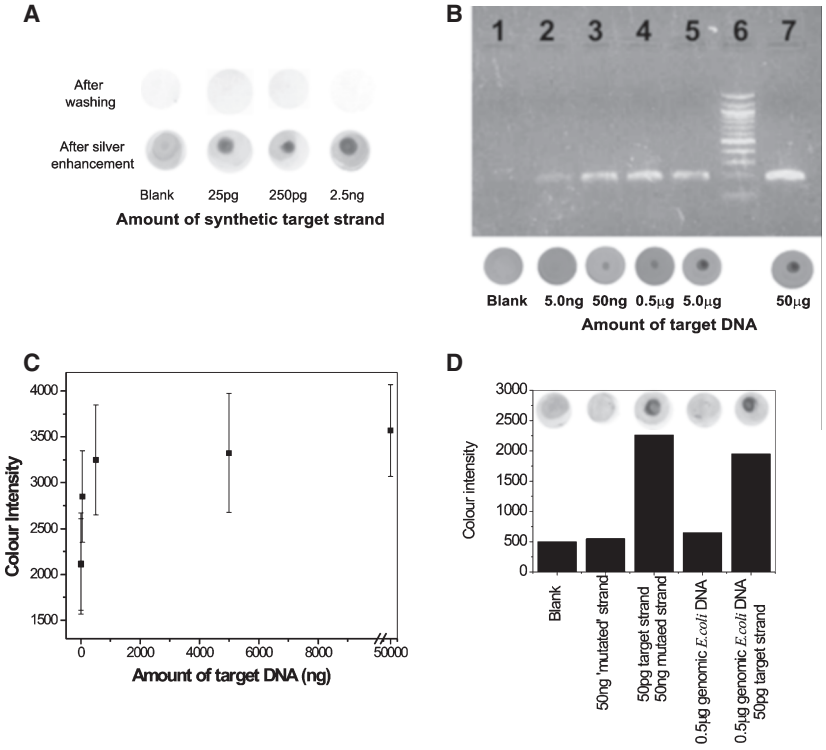

Fig. 2. Examples of results obtained with the gold colloid–latex bead test and PCR. **A**, photographs of filters showing the spots obtained with blank, 25 pg, 250 pg and 2.5 ng synthetic target nucleotide after washing (top row) and silver enhancement (bottom row). The spots are almost invisible after washing but become visible after silver enhancement. **B**, comparison of results obtained with PCR and the gold colloid–latex bead test showing the amplification obtained with 5 ng, 50 ng, 0.5 μg, 5 μg and 50 μg of target DNA (lanes 2, 3, 4, 5 and 7, respectively; lane 1, negative control; lane 6, 100-bp DNA ladder). Two-μL of amplified product was loaded in each lane. Spots obtained with the same amount of DNA are shown below every lane. **C**, variation in the intensity of these spots, showing an average of five spots and the standard deviations. **D**, comparison of the intensity of spots obtained from different types of DNA mixtures. The sensitivity of the test remained unchanged in a mixture of similar DNA or large amounts of random DNA.

tive to detect the target sequence among a large pool of nucleotides.

Use of the test with clinical samples

To investigate its possible use as a diagnostic tool, the test was evaluated with DNA extracted from sputum samples from suspected TB patients. The results are summarised in Table 1. All 18 microscopy-positive samples were also positive by culture, gold colloid–latex bead test and PCR. Of the remaining 69 microscopy-negative samples, 20 were culture-positive, 16 were PCR-positive, and 13 were gold colloid–latex bead test-positive. Of particular interest is the comparison between the gold colloid–latex bead test and PCR, as both tests recognise the same target sequence. Of the 38 culture-positive samples, 32 were PCR-positive, 29 were gold colloid–latex bead test-positive, and 18 were microscopy-positive. None of the culture-negative samples was positive by any of the other methods. Three PCR-positive samples were

Table 1. Summary of results obtained by different methods

Total samples (n = 87)											
Culture-positive (n = 38)						**Culture-negative (n = 48) and contaminated culture (n = 1)**					
Microscopy		**PCR**		**Gold colloid**		**Microscopy**		**PCR**		**Gold colloid**	
+ve	−ve	**+ve**	−ve	**+ve**	−ve	**+ve**	−ve	**+ve**	−ve	**+ve**	−ve
18	20	32	6	29	9	0	49	0	49	0	49

negative by the gold colloid–latex bead test, probably because of its lower sensitivity. The discrepancies with the results of culture could be associated with the limitations of TB diagnosis by detecting the IS*6110* gene [20]. Identical results were obtained using the gold colloid–latex bead test with DNA extracted using either the Chelex 100 procedure (method A) or the boiling method (method B).

Overall, these results demonstrate that the gold colloid–latex bead test can be used to test sputum samples for *M. tuberculosis*. A simplified DNA extraction method is likely to be adequate for the gold colloid–latex bead test, as no amplification occurs and inhibitors have a very limited influence on the results. The sensitivity of this test is lower than that of PCR, but it is simple to perform and the results are visible to the naked eye. The cost of the reagents per DNA extraction and test is 2 Euros, and the test should be suitable for use in field-level laboratories. A further large-scale validation of the test is desirable.

ACKNOWLEDGEMENTS

This work was supported by the core grant from the Department of Biotechnology, Government of India to the National Institute of Immunology, New Delhi. N. Ravat and S.V.K. Anand standardised and performed the PCR assay reported in this paper.

REFERENCES

1. Elghani R, Storhoff JJ, Mucic RC, Letsinger RL, Mirkin CA. Selective colorimetric detection of polynucleotides based on the distance-dependent optical properties of gold nanoparticles. *Science* 1997; **277**: 1078–1081.
2. Reynolds RA, Mirkin CA, Letsinger RL. A gold nanoparticle/latex microsphere-based colorimetric oligonucleotide detection method. *Pure Appl Chem* 2000; **72**: 229–235.
3. Park SJ, Taton TA, Mirkin CA. Array-based electrical detection of DNA with nanoparticle probes. *Science* 2002; **295**: 1503–1506.
4. Wei Y, Cao C, Jin R, Mirkin CA. Nanoparticles with Raman spectroscopic fingerprints for DNA and RNA detection. *Science* 2002; **297**: 1536–1540.
5. Nam JM, Stoeva SI, Mirkin CA. Bio-bar-C code-based DNA detection with PCR-like sensitivity. *J Am Chem Soc* 2004; **126**: 5932–5933.
6. Storhoff JJ, Marla SS, Bao P *et al*. Gold nanoparticle-based detection of genomic DNA targets on microarrays using a novel optical detection system. *Biosens Bioelectron* 2004; **19**: 875–883.
7. Dubertret B, Calame M, Libchaber AJ. Single-mismatch detection using gold-quenched fluorescent oligonucleotides. *Nat Biotechnol* 2001; **19**: 365–370.
8. Storhoff JJ, Lucas AD, Garimella V, Bao YP, Muller UR. Homogeneous detection of unamplified genomic DNA sequences based on colorimetric scatter of gold nanoparticle probes. *Nat Biotechnol* 2004; **22**: 883–887.
9. Li H, Rothberg L. Colorimetric detection of DNA sequences based on electrostatic interactions with unmodified gold nanoparticles. *Proc Natl Acad Sci U S A* 2004; **101**: 14036–14039.
10. Thierry D, Brisson-Noel A, Vincent-Levy-Frebault V, Nguyen S, Guesdon JL, Gicquel B. Characterisation of a *Mycobacterium tuberculosis* insertion sequence, IS*6110*, and its application in diagnosis. *J Clin Microbiol* 1990; **28**: 2668–2673.
11. Andersen AB, Thybo S, Godfrey-Faussett P, Stoker NG. Polymerase chain reaction for detection of *Mycobacterium tuberculosis* in sputum. *Eur J Clin Microbiol Infect Dis* 1993; **12**: 922–927.
12. Lazraq R, el Baghdadi J, Guesdon JL, Benslimane A. Evaluation of IS*6110* as amplification target for direct tuberculosis diagnosis. *Pathol Biol* 1999; **47**: 790–796.
13. Almeda J, Garcia A, Gonzalez J *et al*. Clinical evaluation of an in-house IS*6110* polymerase chain reaction for diagnosis of tuberculosis. *Eur J Clin Microbiol Infect Dis* 2000; **19**: 859–867.
14. Cheng VC, Yam WC, Hung IF *et al*. Clinical evaluation of the polymerase chain reaction for the rapid diagnosis of tuberculosis. *J Clin Pathol* 2004; **57**: 281–285.
15. Danscher G. Localisation of gold in biological tissue. A photochemical method for light and electronmicroscopy. *Histochemistry* 1981; **71**: 81–88.
16. Bhaskar S, Singh S, Sharma M. A single-step immunochromatographic test for the detection of *Entamoeba histolytica* antigen in stool samples. *J Immunol Methods* 1996; **196**: 193–198.
17. Thierry D, Cave MD, Eisenach KD *et al*. IS*6110*, an IS-like element of *Mycobacterium tuberculosis* complex. *Nucleic Acids Res* 1990; **18**: 188.
18. Chakravorty S, Tyagi JS. Novel use of guanidinium isothiocyanate in the isolation of *Mycobacterium tuberculosis* DNA from clinical material. *FEMS Microbiol Lett* 2001; **205**: 113–117.
19. Manabe YC, Dannenberg AM Jr, Bishai WR. What we can learn from the *Mycobacterium tuberculosis* genome sequencing projects. *Int J Tuberc Lung Dis* 2000; **4**: S18–S23.
20. Chauhan A, Chauhan DS, Parashar D *et al*. VMDNA fingerprinting of *Mycobacterium tuberculosis* isolates from Agra region by IS*6110* probe. *Ind J Med Microbiol* 2004; **22**: 238–240.